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We previously identified two human cofactors TIP30 and CIA that can specifically regulate ERα-mediated transcription. TIP30, also called CC3 and Htatip2, is a putative metastasis suppressor that promotes apoptosis and inhibits angiogensis , through the regulation of transcription. TIP30 interacts with an ERα-interacting coactivator CIA (Coativator Independent of AF2). Therefore, we hypothesized that overexpression of both TIP30 and CIA will inhibit transcription of ER-target genes such as c-Myc and Cyclin D1 and overexpression of TIP30 and CIA in ER-negative MDA-MB-231 breast cancer cells. We propose to determine the effects of TIP30 and CIA on expression of ERα-responsive genes, such as c-Myc and Cyclin D1 in the first year. Now, we demonstrate that TIP30 and an CIA are dynamically associated with transcription initiation and elongation complexes in response to estrogen. TIP30 overexpression represses ERα-mediated c-*myc* transcription, whereas TIP30 deficiency enhances *c-myc* transcription in the absence and presence of estrogen. Ectopic CIA cooperates with TIP30 to repress ERα-mediated c-*myc* transcription. In addition, overexpression of TIP30 and CIA in estrogen-independent breast cancer cells suppresses cell growth and induce apoptosis. We suggest that TIP30 and CIA act as negative regulators to control transcription of the *c-myc* and cyclin D1. Our data provides a new pathway for TIP30-mediated tumor suppression and set a stage to study the mechanisms in which this cofactor regulates expression of genes that play important roles in tumorigenesis.

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TITLE: Regulation of Estrogen-Responsive Gene Expression and Tumor Suppression by Transcriptional Cofactors

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Table of Contents

Introduction:

The growth and metastatic potential of breast cancer cells are regulated by estrogen, a ligand of the estrogen receptors (ERs) (1, 2). The ERs facilitate major effects of estrogen in cells through interactions with transcriptional cofactors to regulate transcription of ER-targeting genes. We previously identified two human cofactors TIP30 and CIA (Coativator Independent of AF2) that can specifically regulate ERα-mediated transcription. TIP30, also called CC3 or Htatip2, is a tumor suppressor that can promote apoptosis and inhibits angiogensis, through the regulation of transcription (3-5). TIP30 interacts with an ERα-interacting coactivator CIA (6,7). Therefore, we hypothesized that overexpression of both TIP30 and CIA will inhibit transcription of ER-target genes such as c-Myc and Cyclin D1 and overexpression of TIP30 and CIA in ER-negative MDA-MB-231 breast cancer cells suppresses cell growth. We proposed to determine the effects of TIP30 and CIA on expression of ERα-responsive genes, such as c-Myc and Cyclin D1 in the first year. Now, we show that TIP30 overexpression represses ERα-mediated c-*myc* transcription, whereas TIP30 deficiency enhances *c-myc* transcription in the absence and presence of estrogen. Ectopic CIA cooperates with TIP30 to repress ERα-mediated c-*myc* transcription. Using chromatin immunoprecipitation (ChIP) assays (8), we demonstrate that TIP30 and CIA are distinct cofactors that are dynamically associated with transcription initiation and elongation complexes in response to estrogen. Both TIP30 and CIA are recruited to the *c-myc* gene promoter by liganded ERα in the second transcription cycle. Overexpression of TIP30 and CIA in ER-negative MDA-MB-231 breast cancer cells suppresses cell growth.

Body:

Task 1. **To determine the effects of TIP30 and CIA on expression of ERα-responsive genes, such as c-Myc and Cyclin D1 (months 1-12).**

a. Prepare various plasmid DNA for transient transfection assays (months 1-2)

b. Perform transient transfection and luciferase assays to determine the effects of TIP30 and CIA on transcription of the c-Myc and Cyclin D1 promoter (months 3-9).

c. Perform ChIP assays to determine the occupancy of TIP30 and CIA on the c-Myc and Cyclin D1 gene in vivo (months 10-16)

We have completed this task and the results from this work were included in an article (6) that was in press in Journal of Biological Chemistry 2004 (attached in appendix and JBC online).

Dynamic assembly of TIP30 and CIA to the cyclin D1 promoter. We next asked whether TIP30 and CIA are recruited the promoter of the cyclin D1 gene that is also an ERα-targeting gene. With a ChIP assay, we found that CIA was also assembled on the cycD1 promoter in the presence of E2 (Fig. 1). E2 induced dissociation of TIP30 from the promoter.

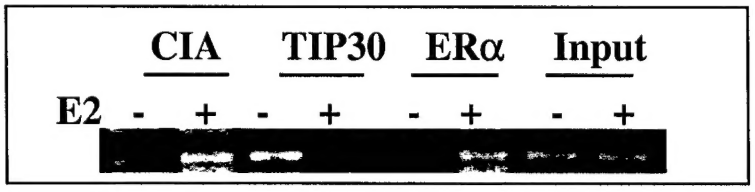

Fig. 1. ChIP assay reveals that TIP30 and CIA are dynamically assembled on the cyclin D1 promoter. ChIP assays were performed as described

above. A pair of primers covering –372 to –30 (containing CRE and AP1 Sites) was used for amplification of DNA.

Task 2. **To determine the effects of SERMs on TIP30 and CIA mediated transcriptional regulation of the c-Myc and the cyclin D1 genes (months 17-20).**

1. Perform transient transfection and luciferase assays to determine the effects of SERMs on TIP30 and CIA mediated transcriptional regulation of the c-Myc and the cyclin D1 genes (months 17-20).
2. Perform ChIP assays to determine the effects of SERMs on recruitment of TIP30 and CIA to the c-Myc and the cyclin D1 genes (months 20-24)

Tamoxifen (TAM) is an antagonist of E2 that inhibits ERα-mediated transcription in MCF-7 cells. This is partly because TAM-bound ERα recruits the N-CoR/SMRT co-repressors to the promoter and prevents recruitment of p160, AIB1, CBP and p300. To investigate whether TAM affects occupancy of TIP30 and CIA on the *c-myc* promoter, MCF-7 cells were treated with TAM and subjected to ChIP analyses. As expected, recruitment of ERα, but not CBP, to the *c-myc* promoter was observed (data was shown in previous report). As was observed with E2, TAM induced association of CIA with the *c-myc* promoter but reduced TIP30 occupancy. This result suggests that CIA, but not TIP30, was present in the TAM-ERα repression complex assembled on the promoter of the *c-myc* gene.

Task 3. **To determine the effects of TIP30 and CIA on proliferation, motility and apoptosis in ER-negative MDA-MB-231 breast cancer cells.**

a. Establish various MDA-MB-231 breast cancer cell lines stably expressing TIP30, TIP30 mutant, CIA or CIA mutant (months 1-6)
b. Measure the growth rates of various cell lines and perform MTT assays to determine rates of DNA synthesis (months 7-12)
c. Measure the capacities of cells to migrate and invade in order to assess the ability of TIP30 and CIA to regulate motility of breast cancer cells (months 13-14).
d. Assay the sensitivity of cells to apopotsis in order to assess the ability of TIP30 and CIA to induce cell death (months 15-20)
e. Develop a series of retroviruses expressing wild type and mutant TIP30 and CIA (months 21- 28)
f. Perform viral infections and assays for growth rates and apoptosis to determine whether ectopic expression of both TIP30 and CIA in breast cancer cells could inhibit cell growth and induce apopotsis (months 28-36)

TIP30 and CIA act synergistically to promote cell death in MDA-MB-231 cells

In order to assess the biological significance of overexpression of TIP30 and/or CIA in the growth of estrogen-independent breast cancer cells, TIP30 and CIA expression vectors were introduced into MDA-MB-231 cells. We first use TIP30 and CIA expression plasmids for making stable cell lines. We found that the efficiency of transient transfection in this cell line was very low using several commercial reagents. To efficiently introduce the expression vectors, we then constructed the pBabe-flagTIP30-puro retroviral vector and pBabe-CIA-puro retroviral expression vector and used 293T

cells to generate replication-deficient viral particles of pBabe-puro, pBabe-flagTIP30-puro, pBabe-CIA-puro, or pBabe-flagTIP30-puro and pBabe-CIA-puro. We have made stable MDA231 cells containing expressing vectors. However, we could not detect either ectopic wild-type TIP30 or ectopic CIA proteins in those cells by Western blot analysis. However, the MDA-MB-231 cells containing mutant TIP30 expression vectors overexpress mutant TIP30 proteins. This result suggests that overexpression of TIP30 or CIA was not well tolerated by these cells. Therefore, a possibility for failure of cells stably overexpressing TIP30 or CIA is that overexpression of TIP30 or CIA may suppress the growth of MDA-MB-231 cells through induction of apopotsis. To test this possibility, we examined the growth of MDA-MB-231 cells in a short-term expression assay by utilizing retroviruses expressing TIP30 and/or CIA in three days. At 48 hours after infection with retroviruses and antibiotic selections, the MDA-MB-231 cells were plated in low serum culture at equal density, and cells were counted after 36 hrs. We found that less than 2% live cells were found in the plates growing MDA-MB-231 cells infected with retroviruses expressing both TIP30 and CIA. Based on a preliminary study, we proposed that ectopic expression of TIP30 could cooperate with CIA to inhibit MDA-MB-231 cell growth. However, in this experiment, it was not clear whether a MDA-MB-231 cell ectopically expressed both TIP30 and CIA. In order to assure that a cell expressed both ectopic TIP30 and CIA, we have created a retrovirus expressing both TIP30 and CIA by constructing the TIP30 and CIA genes into the viral expression vector and inserting a ribosomal binding site (IRE) between the TIP30 and CIA genes that allows translation of both proteins from one mRNA. We examined the growth of MDA-MB-231 cells in a short-term expression assay by utilizing retroviruses expressing TIP30 and/or CIA. At 48 hours after infection with retroviruses and antibiotic selections, the MDA-MB-231 cells were plated in low serum culture at equal density. Proliferation and growth of cells were measured by MTT assay and counted every 24 hours for six days. As shown in Fig. 2, proliferation of cells was

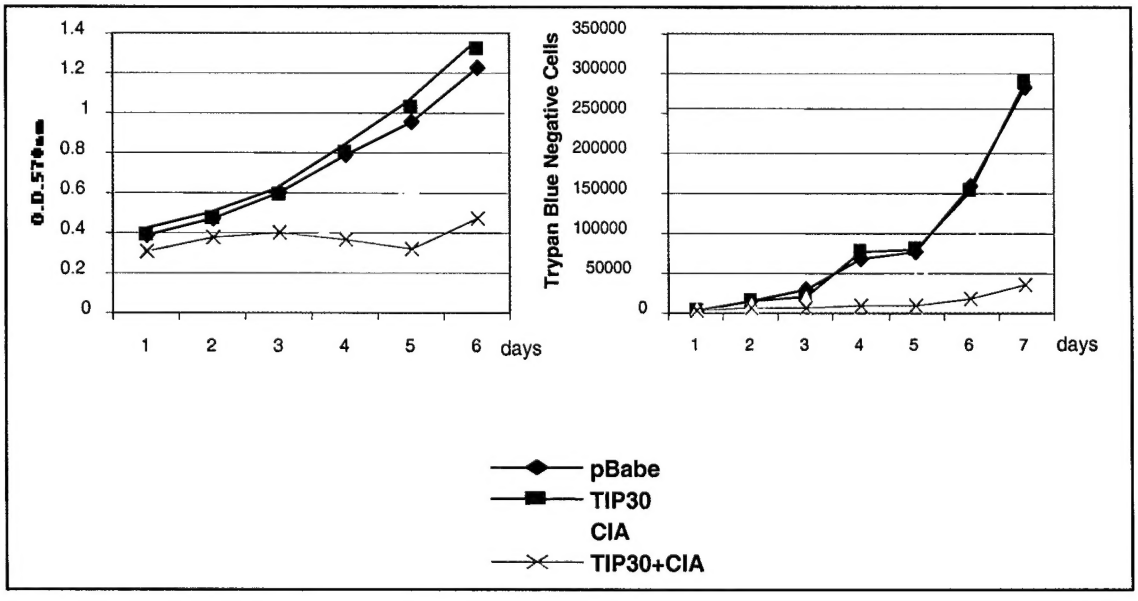

Fig. 2. 293T cells were transfected with empty pBabe-puro vector, pBabe-flagTIP30-

puro, pBabe-CIA-puro, or pBabe-flagTIP30-CIA puro. Virus-containing supernatants were collected 48 hours after transfection. The virus-infected MDA-MB-231 cells were selected in complete media with puromycin for 48 hours and then seeded into the plates. At indicated time points, MTT assay was performed as described and O.D. measurements were plotted (Left panel). At indicated time points, living and dead cells were counted using trypan blue exclusion (right panel). Values shown are average numbers from three experiments.

not significantly affected by retrovirus expressing TIP30 as compared to cells infected with control viruses. In contrast, proliferation of cells was significantly inhibited by retroviruses expressing CIA and completely inhibited by retroviruses expressing both CIA and TIP30. Similarly, MDA-MB-231 infected with control retroviruses or TIP30-expressing viruses grow at a similar rate. This data support our hypothesis that TIP30 and CIA act synergistically to predispose estrogen-independent breast cancer cells to apoptosis.

In summary, we have completed almost all tasks that we proposed to do in the first and second year. The results were partly published in Journal of Biological Chemistry in 2004. In addition, we have done some of the experiments which we proposed to do in the third year. We have not done transient transfection assays for the cyclin D1 promoter, which we are going to do in the third year.

Key research Accomplishments:

Our data suggest that TIP30 acts as a negative regulator for ERα-mediated *c-myc* transcription. Overexpression of TIP30 and CIA in estrogen-independent breast cancer cells suppresses cell growth.

Reportable outcomes:

Part of this work was in press in Journal of Biological Chemistry, 2004 and was included in the preliminary data in a NIH RO1 application (RO1 DK066110-01) that was funded for five years.

Part of this work was presented as a poster at the 2003 AACR Meeting on Breast Cancer. Abstract Title: A tumor suppressor TIP30 regulates c-MYC transcription and mammogenesis.

Part of this work was presented as a post at the AACR Special Conference in Cancer Research. Hyatt Regency Huntington Beach Resort and Spa. Huntington Beach, CA. October 8-12, 2003

A42 Chao Jiang, Mitushiro Ito, Kay-Uwe Wagner, Kyung-Ran Park, Kristy Krumm, Jill Pecha, Robert G. Roeder and Hua Xiao. TIP30 Interacts with an ERα-interacting Coactivator CIA and Regulates c-myc Transcription and Mammogenesis.

Part of this work was presented by Chao Jiang at the AACR 95th Annual Meeting, Orlando, FL. March 27-31, 2004. 5557. The title of her talk was "TIP30 acts as a tumor suppressor in hepatocarcinogenesis".

Conclusions:

We demonstrate that a tumor suppressor TIP30 and an ER-interacting coactivator CIA are dynamically associated with transcription initiation and elongation complexes in response to estrogen. TIP30 overexpression represses ERα-mediated c-*myc* transcription, whereas TIP30 deficiency enhances *c-myc* transcription in the absence and presence of estrogen. Ectopic CIA cooperates with TIP30 to repress ERα-mediated c-*myc* transcription. In addition, overexpression of TIP30 and CIA in estrogen-independent breast cancer cells suppresses cell growth and induces apoptosis. We suggest that TIP30 and CIA act as negative regulators to control transcription of *c-myc* and cyclin D1. Our data provides a new pathway for TIP30-mediated tumor suppression and sets a stage to study the mechanisms in which this cofactor regulates expression of genes that play important roles in tumorigenesis.

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TIP30 Interacts with an Estrogen Receptor α-Interacting Coactivator CIA and Regulates c-*myc* Transcription*

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AQ: A

Deregulation of c-*myc* expression is implicated in the pathogenesis of many neoplasias. Estrogen receptor α (ERα) can increase the rate of c-*myc* transcription through the recruitment of a variety of cofactors to the promoter, yet the precise roles of these cofactors in transcription and tumorigenesis are largely unknown. We show here that a putative tumor suppressor TIP30, also called CC3 or Htatip2, interacts with an ERα-interacting coactivator CIA. Using chromatin immunoprecipitation assays, we demonstrate that TIP30 and CIA are distinct cofactors that are dynamically associated with the promoter and downstream regions of the c-*myc* gene in response to estrogen. Both TIP30 and CIA are recruited to the c-*myc* gene promoter by liganded ERα in the second transcription cycle. TIP30 overexpression represses ERα-mediated c-*myc* transcription, whereas TIP30 deficiency enhances c-*myc* transcription in both the absence and presence of estrogen. Ectopic CIA cooperates with TIP30 to repress ERα-mediated c-*myc* transcription. Moreover, virgin TIP30 knockout mice exhibit increased c-*myc* expression in mammary glands. Together, these results reveal an important role for TIP30 in the regulation of ERα-mediated c-*myc* transcription and suggest a mechanism for tumorigenesis promoted by TIP30 deficiency.

AQ: B

Estrogen plays an important role in the development and maintenance of the mammary glands, as well as various other tissues, and in numerous human diseases that include breast and endometrial cancer, cardiovascular disease, and osteoporosis (1–5). Most of the effects of estrogen are facilitated by estrogen receptor α (ERα),[1] which controls the expression of a number of hormone-responsive genes (5, 6), including the c-*myc* gene, which is important for cell proliferation (7–10). ERα, like many other nuclear receptors, contains two intrinsic transcriptional activation domains, designated AF-1 and AF-2 (3, 11, 12). The function of AF-1 is estrogen-independent, whereas the activity of AF-2 is estrogen-dependent. AF-1 and AF-2 activities show promoter context and cell type specificity and can act synergistically to activate transcription (5).

Fn1

Although the precise mechanisms by which ERα regulates gene expression are still not clearly defined, there is solid evidence suggesting that ERα executes its effects by directing cyclical and combinatorial recruitment of cofactors on promoters (13–17). Most ERα-interacting coactivators identified thus far are also able to interact with many other members of the nuclear receptor superfamily and have generalized functions, hence affecting transcriptional activation mediated by a wide spectrum of nuclear receptors (17–21). More recently, Giguere and co-workers (22) identified a novel nuclear receptor coactivator, called CIA (coactivator independent of AF-2 function). CIA was shown to interact with ERα and ERβ in a ligand-dependent manner but not with other members of the nuclear receptor family. Consistent with its binding specificity, CIA was found to potentiate transcriptional activation by the ER but not by other nuclear receptors (22). Nevertheless, the precise mechanism by which CIA enhances transcription remains unknown. CIA may represent a novel class of ligand-dependent ER coactivators that are independent of AF-2 function.

We previously purified a protein, TIP30 (Tat-interacting protein 30) that interacts specifically with the activation domain of Tat (23). TIP30 is identical to CC3, which is absent in highly metastatic human small cell lung carcinoma (24). TIP30/CC3 has been proposed to function as a metastasis suppressor via its ability to promote apoptosis and inhibit angiogenesis (24–27). Consistent with this hypothesis, ectopic expression of TIP30 was found to elevate the expression of a subset of pro-apoptotic genes (27) and angiogenic inhibitors and to down-regulate the expression of certain angiogenic stimulators (25). Moreover, deletion of one or both alleles of *Tip30* results in spontaneous development of hepatocellular carcinomas and other tumors in mice at a relatively long latency (28). Reduced expression of TIP30 is observed in 33% of human hepatocellular carcinomas, and mutations in the *Tip30* gene that caused the instability or abnormal cellular distributions of the TIP30 protein were identified in some of the human hepatocellular carcinoma specimens (28). These data further suggest that TIP30 is a tumor suppressor.

AQ: C

* This work was supported by American Cancer Society Research Scholar Grant for Beginning Investigators RSG0216501GMC, Department of Defense Breast Cancer Research Program Idea Award DAMD170210507, a University of Nebraska Medical Center start-up grant (to H. X.), and a National Institutes of Health grant (to R. G. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ER, estrogen receptor; MEF, mouse embryonic fibroblast; E2, 17β-estradiol; GST, glutathione *S*-transferase; ChIP, chromatin immunoprecipitation; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TR, thyroid receptor; CBP, CREB-binding protein.

AQ: L

In this study, we describe the cloning and characterization of a TIP30-interacting protein identical to CIA and provide evidence that TIP30 is an important regulator of ERα-mediated c-*myc* transcription. This study defines a new pathway for regulating expression of the c-*myc* gene and possibly other ERα target genes that are involved in tumoriogenesis.

EXPERIMENTAL PROCEDURES

Protein Purification and Chromatography—The HeLa cell line (HeLa-fTIP30) stably expressing FLAG-tagged TIP30 was obtained by the introduction of pCIN4-FLAG-tagged TIP30 into HeLa S3 and selection in a G418-containing medium (27). Nuclear extracts were prepared as described previously (29) with the following modifications. During purification procedures, HEPES-HCl, pH 7.9, was used in buffers instead of Tris-HCl. Nuclear pellets were resuspended in a low salt buffer containing sulfosuccinimidylpropionate (Pierce), transferred to a homogenizer, and mixed with six strokes of a loose pestle. The suspensions were then transferred to glass beakers and dispersed with a stirring bar. The high salt buffer was slowly added into the mixtures. The cross-linking reactions were incubated at 4 °C for 2 h and then stopped by adding 1 M Tris-HCl, pH 8.0, to a final concentration of 20 mM. The extracts were centrifuged at 15000 rpm for 30 min and stored at −80 °C. For coimmunoprecipitation, 100 ml of nuclear extract prepared from the HeLa-f:TIP30 cells was mixed with 200 μl of anti-FLAG epitope immunoaffinity matrix M2 (Sigma) in BC450 containing 0.1% Nonidet P-40 and 0.45 M KCl. The matrix was washed extensively with BC500 plus 0.1% Nonidet P-40 and 0.5 M KCl and eluted with 1 ml of BC1000 containing 200 μg/ml FLAG peptide and 1 M KCl. The eluted proteins were concentrated and resolved on SDS-PAGE and stained with silver or Coomassie Blue. The indicated bands were excised from the gel and digested with endoproteinase C. The resulting peptides were isolated by high pressure liquid chromatography and subjected to microsequencing analysis (Core Facility, Rockefeller University). Affinity chromatography was carried out as described previously (23).

Cloning of the CIA Gene—An expressed sequence tag clone (DKFZp434J208) was initially identified by data base search using two peptides (NMPQADAMVLVAR and DLRDFR) from amino acid sequencing and peptide mass data from mass spectrophotometer analysis of a TIP30-interacting protein (65 kDa). The expressed sequence tag clones (IMAGE: 1185534 and 1335209), containing similar cDNA sequences, were identified and purchased from ATCC and sequenced. Primers were designed for 5′-RACE and 3′-RACE PCRs according to the sequence of the insert cDNA. The HeLa cDNA library (Clontech) was used as a template for 5′-RACE and 3′-RACE. The experiments were carried out according to the manufacturer's instruction (Clontech). The resulting DNA fragments were subcloned into a pCR2.1 vector by PCR kits (Invitrogen). Several cDNA fragments were amplified and sequenced by the core facility at the University of Nebraska Medical Center, and the resulting sequences were used for searching the expressed sequence tag clones and known genes in GenBank™. The 856-bp cDNA containing the translational start codon was amplified using PCR primers (primary primer, 5′-ATCTCTACTATGTGTGTGGTCCCG; nest primer, 5′-CAAGTCTCGGGGGTCTCGAATGTC). The pCR2.1-CIA containing full-length CIA cDNA was constructed by subcloning a PCR-amplified cDNA from the HeLa cDNA library (Clontech) using specific primers (forward, 5′-GGAAGATCTATGAATACGGCTCCATCAAGACCCAGC; reverse, 5′-GGAAGATCTCAGTAATGCCTCTGGTAAGATCCCAT).

Plasmids and Antibodies—The pcDNA3.1-CIA plasmid was generated by inserting the CIA cDNA, excised from pCR2.1-CIA with BglII, into pcDNA3.1 at the BamHI site. For antibody production, the bacterial expression plasmid pREST-his-CIA was generated by inserting a BglII-DNA fragment released from pCR2.1-CIA into pRSET-B (Invitrogen). The recombinant His tag CIA protein was expressed in bacteria, purified as described previously, and used to generate rabbit anti-CIA antibodies (Covance Inc., Denver, PA). The pGL3-hu-Myc plasmid containing the human c-*myc* promoter was constructed by subcloning a 2540-bp HindIII-DNA fragment excised from pSV40CAT-hu-Myc plasmid (a gift from David Bentley, University of Colorado Health Science Center) into pGL3-basic vector (Promega) at the HindIII site. This DNA fragment extends 2.3 kb 5′ of the P1 start site to a NaeI site at +50 relative to the P2 start site. Antigen-purified anti-TIP30 (23) and anti-CIA antibodies were purified as described previously (30). Anti-ERα (HC-20, Santa Cruz Biotechnology), anti-CBP (A22, Santa Cruz Biotechnology), and anti-RNA polymerase II (8WG16) antibodies were used for ChIP assays.

Mice and MEFs—Generation of TIP30 null mice and MEFs was described previously (28). The genetic backgrounds of *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice were regarded to be identical because they were backcrossed 10 times with C57BL6J mice. MEF clones of each genotype were prepared from sibling embryos obtained by a heterozygous crossing. All animal experimentation was performed according to the National Institutes of Health guidelines.

Pull-down Assay—Pull-down assays were performed as described previously (22). [^{35}S]Methionine-labeled CIA and Bax proteins were generated by *in vitro* transcription and translation using the TNT-coupled reticulocyte lysate system (Promega). Fusion proteins were induced in DH5α *Escherichia coli* with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 2 h. The cells were sonicated 10 times using 30-s pulses in 1× phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. The cleared lysates were bound to glutathione-Sepharose (Amersham Biosciences) for 15 min at 4 °C. The protein-saturated beads were washed three times in 1× phosphate-buffered saline with 1 M NaCl and stored in BC100 (20 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 100 mM KCl, 0.1% Nonidet P-40). 20 μl of GST or GST-TIP30 beads were incubated with 10 μl of [^{35}S]methionine-labeled proteins in 10 μl of BC135 buffer (20 mM Tris-HCl, pH7.9, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1% Nonidet P-40, 135 mM KCl) for 2 h at 4 °C. The binding reaction mixture was washed three times in BC135 buffer. The bound proteins were analyzed by SDS-PAGE followed by autoradiography of fixed and dried gels.

ChIP Assays—ChIP assays were performed as described previously (15). MCF-7 cells were treated with or without 10^{-8} M E2 for 45 min and with formaldehyde to cross-link proteins to DNA. Soluble chromatin was prepared and immunoprecipitated with preimmune serum and protein A-Sepharose and then immunoprecipitated with anti-CIA, anti-TIP30 serum, or control anti-ERα, anti-CBP, and anti-RNA polymerase II antibodies. The final DNA preparations were amplified using a pair of primers and analyzed in 1.5% agarose gel following ethidium bromide stain.

Transient Transfection—Transfection assays were performed as described previously (26). COS-1 cells in 24-well dishes or MEFs in 6-well dishes were transfected with plasmid DNA using LipofectAMINE according to the manufacturer's instruction (Invitrogen). In all experiments, a plasmid pRL-CMV for expressing *Renilla* luciferase was used as a control for transfection efficiency, and activities of firefly and *Renilla* luciferases were measured with Promega's dual luciferase reporter assay system, normalized, and expressed as relative luciferase light units. The vector pCMV-ERα for expressing human ERα and vector pNT7-TRα for human TRα were described previously (31). TRE-luciferase reporter was described previously (31). Gal-RXR expressing vector and Gal-luciferase reporter were described previously (19).

Histology and Immunohistochemistry—Mammary gland 4 was removed and fixed with 10% buffered-formalin and embedded in paraffin blocks. The sections were deparaffinized, rehydrated, and stained with hematoxilin and eosin. For immunohistochemistry staining, unstained sections were rehydrated and incubated overnight at 4 °C with anti-Myc antibody (Upstate Biotechnology, Inc.), and staining was developed using VECTASTAIN Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions and then counter-stained with hematoxilin and eosin. The number of Myc nuclear positive epithelial cells was divided by the total number of epithelial cells counted.

RNA Isolation and RT-PCR—The abundance of c-*myc* mRNA in the tissue was studied by a semi-quantitative RT-PCR analysis using β-actin mRNA as control. Total RNA was isolated from mammary glands of 8-week-old virgin mice using Trizol reagent. 2 μg of DNA-free total RNA was reverse transcripted into cDNA using oligo(dT)$_{12-18}$ and SuperScript II RT (Invitrogen) following the manufacturer's instructions. The same amounts of resulting cDNA were used for PCR amplification. The primers used for PCR and their sequences are as follows: c-*myc*: sense, 5′-ATTGAGCCAAATCTTAAGTTGTGA, and antisense, 5′-TTTGGAGTGAGCAGGGGACTGGCA; β-actin: sense, 5′-CACCCTGTGCTGCTCACCGAGGCC, and antisense, 5′-CCACACAGAGTACTTGCGCTCAGG; and cyclin B1: sense, 5′-ACCTACAGGGTCGTGAAGTGACTGGAAAC, and antisense, 5′-TGAGAATCTTCATCTCCATCTGTCTG. The PCR product was analyzed in 1.2% agarose gel following ethidium bromide stain.

RESULTS

Identification of a TIP30-interacting Protein and Cloning Its Full-length Cognate cDNA—The discovery of roles of TIP30 in the control of expression of genes involved in apoptosis and angiogenesis indicates that TIP30 may interact with transcrip-

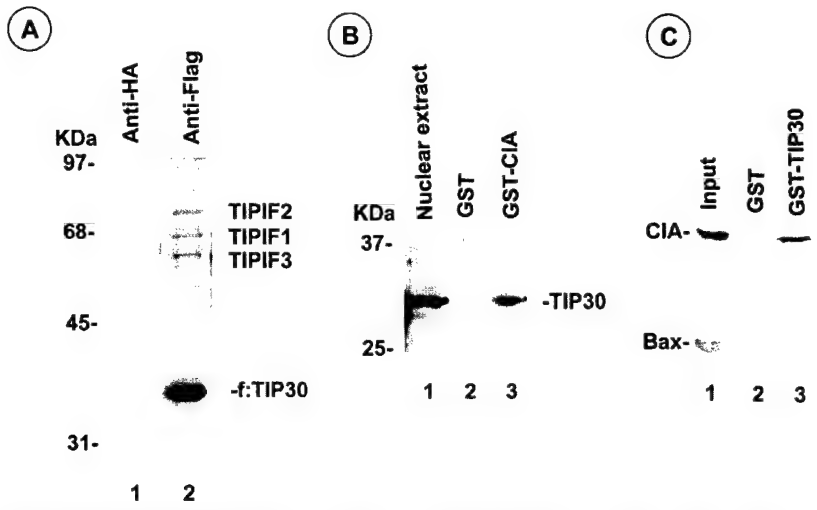

FIG. 1. **Purification of TIP30-interacting proteins.** *A*, TIP30-interacting proteins were purified from HeLa nuclear extracts as described under "Experimental Procedures." The final preparations were resolved in 10% SDS-PAGE and stained with silver (*lane 2*). These three proteins were not coimmunoprecipitated if anti-hemagglutinin epitope immunoaffinity matrix was used (*lane 1*). *B*, CIA binds to TIP30 from HeLa nuclear extracts. 300 μl of HeLa nuclear extracts was chromatographed on 30 μl of GST and GST-CIA columns. The columns were washed with BC100 extensively and eluted with 120 μl of BC1000. The proteins eluted from the columns were separated with SDS-PAGE and immunoblotted with anti-TIP30 antibodies. *C*, TIP30 interacts with *in vitro* synthesized CIA. Pull-down assays were performed as described under "Experimental Procedures." The bound proteins were analyzed by 12.5% SDS-PAGE and autoradiography. The input represents 17% of the [^{35}S]methionine-labeled CIA and [^{35}S]methionine-labeled BAX used in the assay (*lane 1*). CIA, but not a pro-apoptotic protein BAX, interacts with GST-TIP30 (*lane 3*) but not with GST (*lane 2*).

tion factors to regulate gene expression. To identify and affinity purify cellular proteins that interact with TIP30, we first established HeLa cell lines that stably expressed FLAG-tagged wild type TIP30. After preparation of nuclei from these cells, nuclear proteins were cross-linked by mixing the nuclei with the water-soluble cross-linker sulfosuccinimidylpropionate. Sulfosuccinimidylpropionate-treated nuclear extracts were made and subjected to affinity purification with anti-FLAG M2 beads. The bound proteins were eluted with FLAG peptide and analyzed by SDS-polyacrylamide gel electrophoresis after cleaving the cross-linker. As shown in Fig. 1*A*, three proteins were coimmunopurified with TIP30 and designated TIP30-interacting proteins. These three proteins were not immunoprecipitated with control anti-hemagglutinin beads. Amino acid sequencing of one of these interacting proteins (65 kDa), designated TIP30-interacting protein 1 (TIPIF-1), yielded two peptide sequences (NMPQADAMVLVAR and DLRDFR). A HeLa cell-derived 2.2-kb cDNA encoding TIPIF-1 was cloned and sequenced (GenBank™ accession number AF470686). Sequence analysis revealed that it encodes a 587-amino acid protein with a tract of Arg-Asp residues in the amino-terminal region. The repeat Arg-Asp residues were previously identified in the splicing factor U1 70K and the RD protein, a component of the negative elongation factor NELF (32). TIPIF-1 also contains a receptor-binding motif known as the LXXLL motif (33, 34) and seven repeats of a short sequence motif (RDLRD(H/F)R) that is present in subunit 10 of mouse translation initiation factor 3. Following cloning of the TIPIF-1 cDNA, a data base search revealed identity with the estrogen receptor coactivator CIA (22). The reported CIA cDNA, which was isolated from a human fetal kidney cDNA library, contains a 620-residue open reading frame lacking a translational stop codon at the 5′ end. In contrast, the open reading frame in our TIFIF-1 cDNA starts at a methionine residue located immediately after two in-frame stop codons, indicating that it encodes a full-length CIA. The sequence discrepancy in the 5′-untranslated region between TIPIF-1 and CIA may be due to alternative splicing products in HeLa and kidney cells, because the cDNA encoding CIA was cloned from the human fetal kidney cDNA library (22). We now refer to TIPIF-1 as CIA. To ascertain whether CIA interacts with TIP30, we tested the binding of TIP30 from nuclear extracts to a GST-CIA fusion protein. As shown in Fig. 1*B*, TIP30 was detected in the eluate from the GST-CIA affinity column but not in the eluate from the control GST column. In addition, we also tested the binding of *in vitro* translated CIA to GST-TIP30 using a pull-down assay. As shown in Fig. 1*C*, CIA bound GST-TIP30 but not GST control protein. By contrast, BAX protein, a pro-apoptotic protein used as a negative control was not bound by GST-TIP30. Collectively, these results indicate that CIA interacts with TIP30.

Estrogen Regulates TIP30 and CIA Occupancy of the c-myc Gene in Breast Cancer MCF-7 Cells—Transcription of the c-*myc* gene is controlled by transcription factors that interact with numerous positive and negative regulatory elements in the c-*myc* promoter regions (35). ERα can stimulate c-*myc* transcription by interacting with an estrogen-responsive element of the c-*myc* promoter (8). Using ChIP assays, a previous study has established that a number of ERα-interacting coactivators are recruited to the promoters of endogenous estrogen-responsive target genes, including c-*myc*, following estrogen treatment (15). If CIA and TIP30 are specific cofactors for ERα, they might also be associated with ERα on endogenous estrogen-responsive target genes. To test this possibility, we used the same ChIP assay (15) to determine whether CIA and/or TIP30 is recruited to the promoter of the c-*myc* gene in the estrogen-dependent human breast cancer cell line MCF-7. Fig. 2*B* shows that, as previously reported, the anti-ERα antibody precipitated a c-*myc* promoter fragment containing P1 and P2 promoters in the presence (*lane 6*) but not in the absence (*lane 5*) of E2. In contrast, the c-*myc* promoter was precipitated by anti-TIP30 antibody (*lane 3*) but much less so by anti-CIA antibody (*lane 1*) in the absence of E2. E2 increased the association of CIA (*lane 2*) but diminished the association of TIP30 (*lane 4*) with the same DNA region.

Because previous studies demonstrated that ERα and coactivators (AIB1, p300, CBP, pCAF, and TRAP220) are assembled on the promoter during preinitiation but subsequently released during elongation (15), we next determined whether CIA and TIP30 occupy the c-*myc* gene during elongation. Four pairs of primers covering a region (+2196 to +2628) in exon 2

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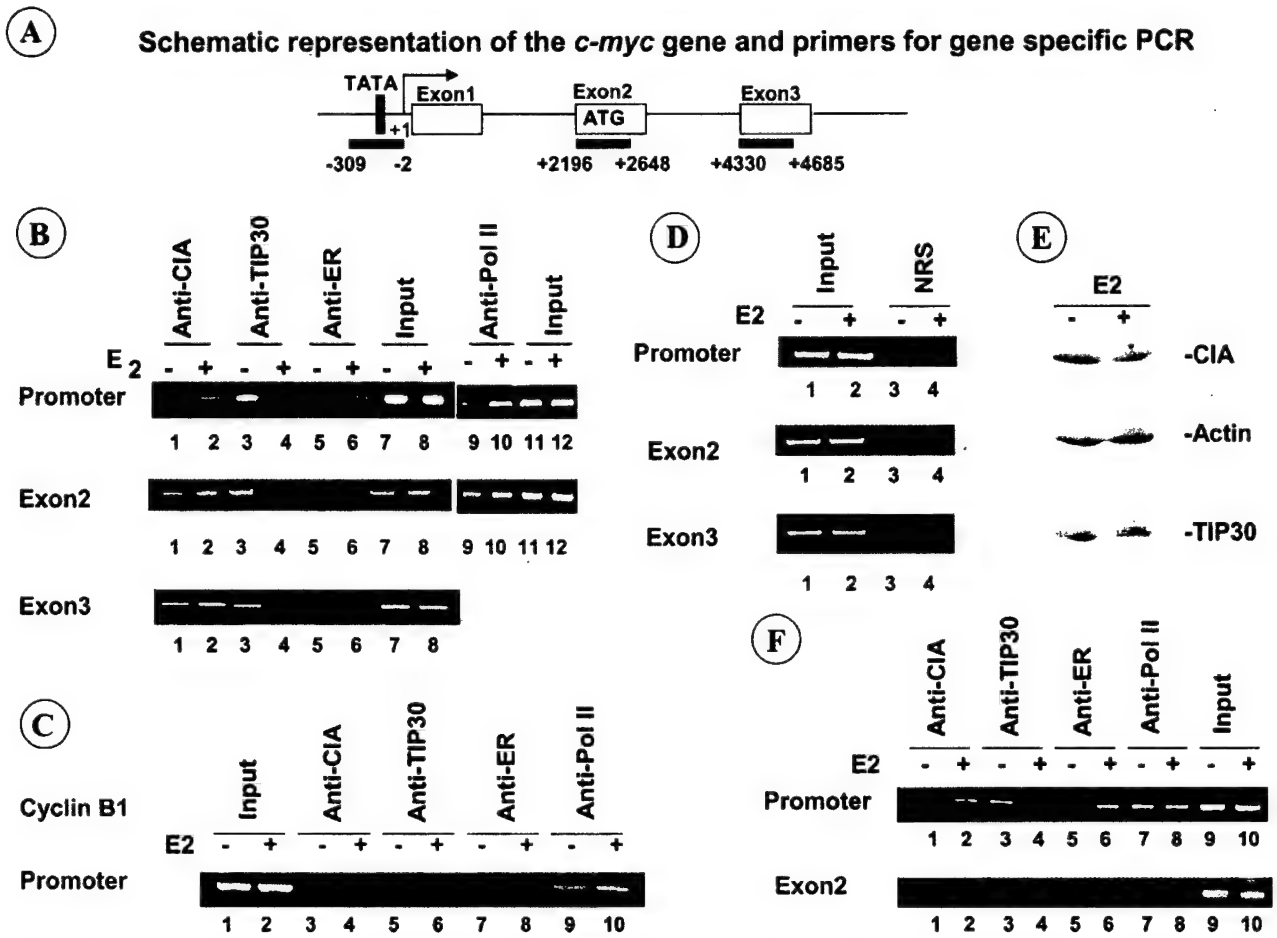

FIG. 2. **Recruitment of CIA and TIP30 to the endogenous c-*myc* gene.** *A*, schematic representation of the c-*myc* gene and primers for PCR. Transcription initiation site and start codon are indicated. The *solid bars* and *numbers* indicate the positions of the primers corresponding to the regions of the c-*myc* gene. The end of c-*myc* mRNA is at position 5190 or 5350 (7). *B*, assembly of CIA and TIP30 on the c-*myc* gene in the first transcription cycle upon E2 induction. MCF-7 cells were treated with 10^{-8} M E2 for 45 min. *Top panel*, recruitment of ERα and CIA to the promoter of the c-*myc* gene. The 307-bp amplified DNA fragment corresponds to the region of the promoter. *Middle panel*, association of CIA with exon 2 that is 2 kb downstream of the initiation site; *bottom panel*, or exon 3 that is 4 kb downstream of the initiation site. The 452- or 355-bp amplified DNA fragments correspond to the region of exon 2 or exon 3, respectively. *C*, ERα, TIP30, and CIA are not assembled on *Cyc B1* promoter. Primer pairs covering −75 to +185 region (33) were used for ChIP analysis. *Pol II*, polymerase II. *D*, ChIP analysis with a normal rabbit preimmune serum (*NRS*). *E*, CIA and TIP30 protein levels in MCF-7 cells in MCF-7 cells after E2 treatment. The cell lysates were prepared from MCF-7 cells treated with or without E2 for 45 min and then analyzed by Western blotting with anti-TIP30, anti-CIA, or anti-β-actin antibodies. *F*, occupancy of TIP30 and CIA on exon 2 is inhibited by α-amanatin. ChIP analysis was performed after cells were treated with 10 μg/ml of α-amanatin for 1 h before the addition of E2.

and a region (+4330 to +4685) in exon 3 were used for PCR amplification of the final DNA preparations. Binding of both TIP30 and CIA to these regions was observed in the absence of E2 (Fig. 2*B*, *lanes 1* and *3*). E2 induction resulted in increased CIA binding (*lanes 1 versus* 2) but decreased TIP30 binding (*lanes 3 versus* 4) to these regions. As a positive control, binding of the largest subunit of RNA polymerase II to the regions upstream and downstream of the initiation site was also observed in both the absence and presence of E2 (Fig. 2*B*, *lanes 9* and *10*). These results suggest that estrogen increases association of CIA with the coding regions of c-*myc*.

To assess the specificity of association of TIP30 and CIA with the c-*myc* gene, we examined whether TIP30 and CIA were assembled on the promoter of the cyclin B1 gene that is not directly regulated by ERα (21). As expected, RNA polymerase II is associated with the promoter (Fig. 2*C*, *lanes 9* and *10*), but ERα is not (*Lanes 7* and 8). However, ERα, TIP30, and CIA were not associated with the promoter in either the presence or the absence of E2 (*Lanes 3*-6). In addition, nonspecific antibodies from a preimmune rabbit serum did not precipitate the DNA elements of the c-*myc* gene (Fig. 2*D*). The protein levels of TIP30 and CIA in MCF-7 cells are not significantly changed after E2 treatment (Fig. 2*E*), suggesting that E2-regulated association of TIP30 and CIA with the c-*myc* gene is not due to the influence of TIP30 and CIA expression by E2. Therefore, associations of these proteins with the c-*myc* gene are specific.

To determine whether the occupancy of the c-*myc* gene by TIP30 and CIA requires elongating RNA polymerase II in estrogen-dependent and -independent transcription, MCF-7 cells were treated with α-amanitin, which specifically inhibits RNA polymerase II elongation (15, 36), and then subjected to ChIP analysis. Consistent with the preceding observations, the occupancy of the c-*myc* promoter by TIP30 and CIA was not affected by α-amanitin (Fig. 2*F*). In contrast, the occupancy of the coding region of the c-*myc* gene by TIP30 and CIA was inhibited by α-amanitin (Fig. 2*E*). This result suggests that association of TIP30 and CIA with the coding regions of the c-*myc* gene depends on elongating RNA polymerase II.

Estrogen Dynamically Regulates Occupancy of the c-myc Gene by TIP30 and CIA—Occupancy of ERα and RNA polymerase II on the promoter was previously shown to peak at 30–45 min in the first transcription cycle and at 120–150 min

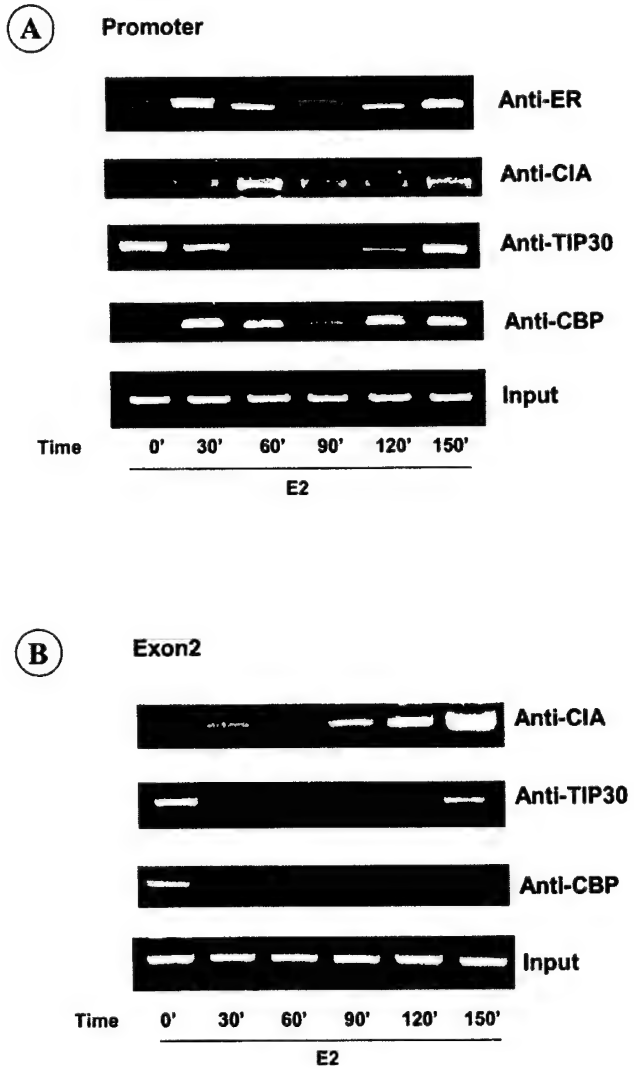

FIG. 3. **Occupancy of the c-*myc* gene by TIP30 and CIA in the second cycle transcription as measured by ChIP.** MCF-7 cells were treated with 10^{-8} M E2 and harvested at different time points. Antibodies used for immunoprecipitation are indicated. *A*, assembly of TIP30 and CIA on the promoter in the second transcription cycle. The 307-bp amplified DNA fragment corresponds to the region of the promoter. *B*, assembly of TIP30 and CIA on exon 2 in the second transcription cycle. The 452-bp amplified DNA fragment corresponds to the region of exon 2.

in the second cycle following the addition of E2 (15). We therefore sought to determine the timing of TIP30 and CIA occupancy on the promoter. As was shown previously, ERα and CBP occupancy on the promoter peaked at 30–60 min in the first transcription cycle and at 150 min in the second cycle upon E2 treatment (Fig. 3*A*). The results obtained with CIA were similar to those obtained with ERα and CBP. Interestingly, TIP30 was dissociated with the promoter at 60 min and reassociated with the promoter at 120–150 min in the second cycle (Fig. 3*A*). Increased binding of TIP30 and CIA to exon 2 was observed at 150 min following E2 treatment (Fig. 3*B*). In contrast, much less CBP was detected to associate with exon 2 upon E2 induction (Fig. 3*B*). Therefore, these results suggest that estrogen increases the recruitment of CIA to but decreases dissociation of TIP30 from the c-*myc* promoter region during the first transcription cycle. Unlike the other known ERα coactivators, which are released during elongation, CIA shows an increased association with the coding regions following E2 treatment. However, in the second cycle of estrogen-induced transcription, both CIA and TIP30 are associated with the promoter as was observed for ERα and CBP. Prolonged estrogen treatment even further increases association of CIA and TIP30 with the coding region of the c-*myc* gene (Fig. 3*B*).

Ectopic Expression of CIA and TIP30 Inhibits ERα-mediated Transcription—To test whether CIA and TIP30 function as coregulators of ERα in the transcription of the c-*myc* gene, we transfected COS-1 cells with vectors expressing ERα, CIA, TIP30, and a kinase-defective mutant TIP30 (30) with a construct containing a luciferase reporter gene controlled by the c-*myc* promoter (−2.3 kb 5′ of P1 start site to +50 relative to P2 start site). As shown in Fig. 4*A*, ectopic TIP30 inhibited the E2-dependent transcriptional activity of ERα in COS-1 cells, whereas ectopic CIA had no significant effect on transcription. The kinase-defective mutant TIP30 (30) did not significantly affect ERα-dependent transcription. Surprisingly and paradoxically, coexpression of ectopic CIA with ectopic TIP30 did not reverse but instead further potentiated the inhibitory effect of TIP30 (4-fold) in ERα-mediated transcription.

To determine whether TIP30 and CIA also regulate transcription from other promoters, we performed transient-transfection experiments using the luciferase reporter gene construct containing the artificial promoter either with estrogen receptor-binding sites (EREs) or with thyroid receptor (TR) α-binding sites (31). As expected, CIA potentiated ERα-mediated transcription from this promoter in the presence of E2 (Fig. 4*B*), but TIP30 inhibited the activity of ERα, whereas a kinase-defective mutant TIP30 (30) did not. Consistent with the observations for c-*myc* promoter, the activity of ERα on the ERE-binding promoter was inhibited by ectopic expression of both TIP30 and CIA. In contrast to ERα, the activity of TRα on the TRE-binding promoter was only slightly increased by cotransfection of TIP30 and/or CIA with TRα (Fig. 4*C*), suggesting that neither TIP30 nor CIA had a significant effect on TR-mediated transactivation.

Taken together, these data indicate that both CIA and TIP30 are important interacting factors in modulating the activity of ERα, and CIA can cooperate with TIP30 to repress ERα-mediated transcription when they are overexpressed. However, because Western blot analyses revealed that the levels of transiently expressed ectopic TIP30 and CIA were much higher than the levels of endogenous TIP30 and CIA in COS-1 cells (data not shown), it is possible that the effects of TIP30 and CIA on ERα-mediated transcription observed here may not represent their physiological functions.

Loss of TIP30 Increases ERα-mediated Transcription—To verify that the effect of TIP30 on ERα-mediated transcription is not limited by its overexpression, we next examined whether the loss of TIP30 expression affected ERα-mediated transcription by performing transient transfection assays in *Tip30*$^{+/+}$, *Tip30*$^{+/-}$, and *Tip30*$^{-/-}$ MEFs. Consistent with the preceding results, Fig. 5*A* shows that the loss of TIP30 results in an increased ERα-mediated transcription from the artificial ERE-binding promoter containing three EREs (*left panel*). Coexpression of either TIP30 alone or TIP30 and CIA in *Tip30*$^{-/-}$ MEFs results in inhibition of ERα-mediated transcription (*right panel*). In contrast, the loss of TIP30 had a minimal effect on ligand-dependent transactivation by Gal4-RXRα (AF2 domain of retinoid X receptor) (19), implying that TIP30 may preferentially inhibit the transcriptional activity of ERα (Fig. 5*C*). Expression of TIP30 did not completely restore ERα activity in *Tip30*$^{-/-}$ MEFs. We reasoned that increased ERα-mediated transcription was partly due to TIP30 deficiency in *Tip30*$^{-/-}$ MEFs and partly due to other genetic changes that these cells may have undergone.

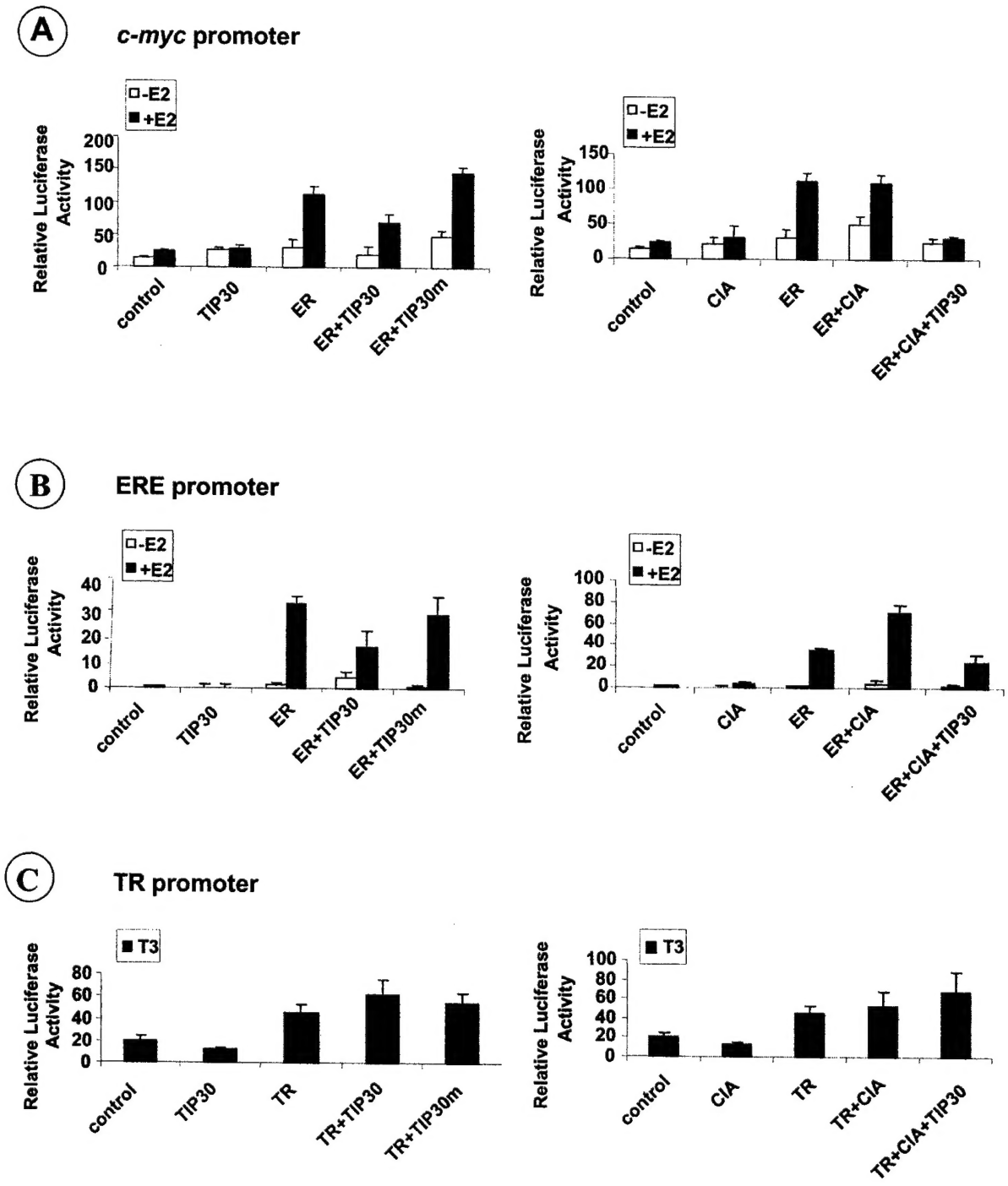

FIG. 4. **Effect on ERα-mediated transcription by overexpression of CIA and TIP30.** *A*, COS-1 cells were transfected with a Myc-luciferase reporter containing the human c-*myc* promoter (250 ng) and vectors for expressing ERα (25 ng), CIA (50 ng), TIP30 (50 ng), or a kinase defective TIP30 mutant (TIP30M, 50 ng) in the presence of 10^{-8} M E2. *B*, COS-1 cells were transfected with a reporter (250 ng) containing three copies of ERE-binding sites and vectors for expressing ERα (25 ng), CIA (50 ng), TIP30 (50 ng), or TIP30m (950 ng), a kinase-defective mutant (27) in the presence of 10^{-8} M E2 as indicated. *C*, COS-1 cells were transfected with a TRE_5-luciferase reporter (250 ng) containing five copies of TRE-binding sites and vectors for expressing human TRα (25 ng), CIA (50 ng), TIP30 (50 ng), or TIP30m (50 ng) as indicated in the presence of 10^{-7} M T3.

We also tested whether the complete absence of TIP30 in cells affects the transcriptional activity of ERα on the c-*myc* promoter. As expected, ERα showed a greater stimulation of the c-*myc* promoter in *Tip30*$^{-/-}$ MEFs than in *Tip30*$^{+/+}$ MEFs (16-fold; Fig. 5*B*, *left panel*) and *Tip30*$^{+/-}$ MEFs (3-fold lower) in the presence of E2. TIP30 loss also resulted in a 5-fold increase in estrogen-independent transcription from the c-*myc* prompter (Fig. 5*B*, *left panel*). This effect was due to the absence of TIP30, because expression of TIP30 in *Tip30*$^{-/-}$ cells resulted in a 42% inhibition in transcriptional activity of ERα (Fig. 5*B*, *right panel*). The finding that deletion of the *Tip30* gene elicits higher transcription from the c-*myc* promoter indicates that the function of TIP30 is to repress both estrogen-independent and estrogen-dependent ERα-mediated c-*myc* transcription.

Lack of TIP30 Increases c-myc Expression in the Mammary

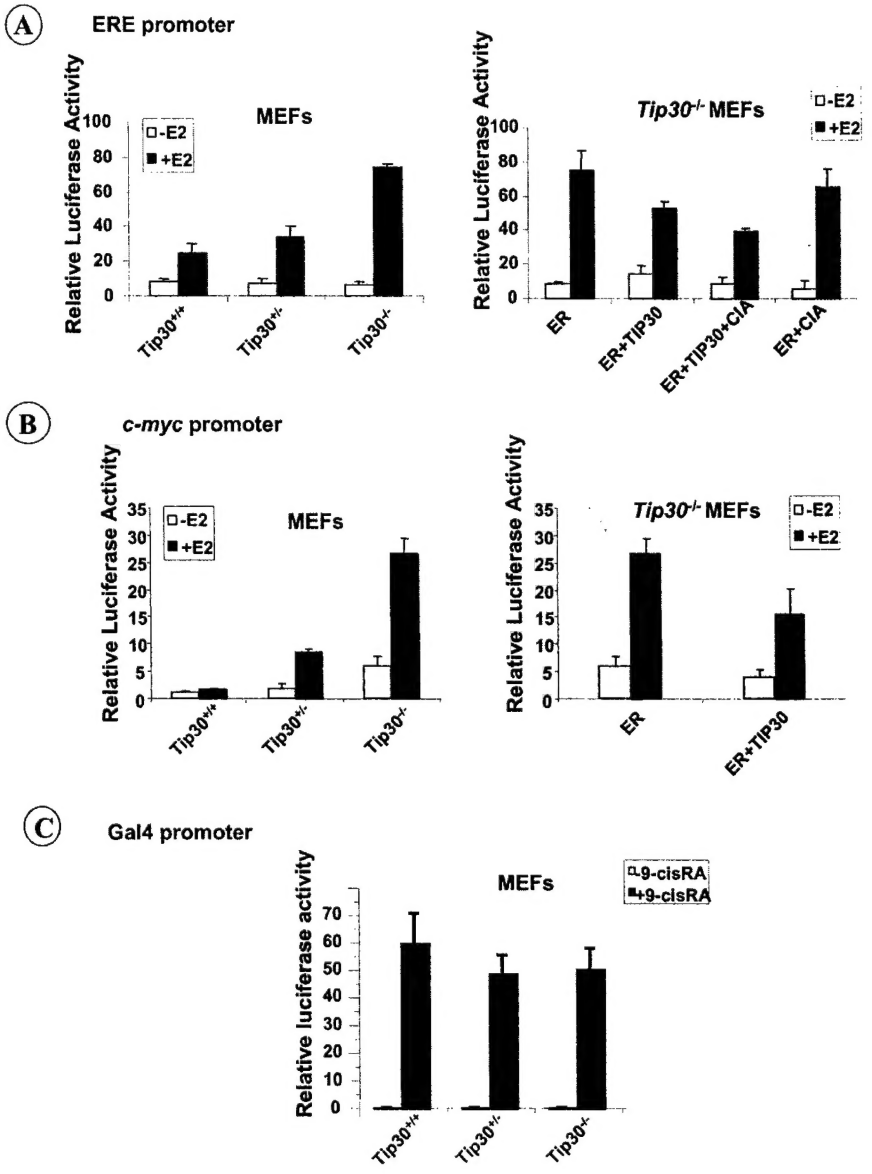

FIG. 5. **Effect on ERα-mediated transcription by loss of TIP30.** *A*, TIP30 loss affects the activity of ERα on an artificial promoter. *TIP30*$^{+/+}$, *TIP30*$^{+/-}$, and *TIP30*$^{-/-}$ MEFs were transfected with ERE-luciferase reporter plasmid and the expression vector for ERα in the presence of either 10^{-8} M E2 or ethanol carrier (*left panel*). TIP30$^{-/-}$ MEFs were cotransfected with ERα, TIP30, and CIA in the presence of either 10^{-8} M E2 or ethanol (*right panel*). *B*, deletion of the TIP30 gene affects the activity of ERα on c-*myc* promoter. *TIP30*$^{+/+}$, *TIP30*$^{+/-}$, and *TIP30*$^{-/-}$ MEFs were transfected with Myc-luciferase reporter and the expression vector for ERα in the presence of either 10^{-8} M E2 or ethanol carrier (*left panel*). TIP30$^{-/-}$ MEFs were cotransfected with ERα and TIP30 in the presence of either 10^{-8} M E2 or ethanol (*right panel*). *C*, Gal4-RXR-driven transcription is not affect by TIP30 loss. *TIP30*$^{+/+}$, *TIP30*$^{+/-}$, and *TIP30*$^{-/-}$ MEFs were transfected with a Gal-luciferase reporter and Gal4-RXR in the presence or absence of 9-*cis*-retinoic acid (*9-cisRA*, 10^{-6} M).

Gland—To demonstrate that TIP30 negatively regulates expression of the c-*myc* gene *in vivo*, we utilized a semi-quantitative RT-PCR analysis to examine the level of c-*myc* mRNA in the mammary gland from virgin *Tip30*$^{-/-}$ mice. As expected, a higher level of c-*myc* RNA was observed in mammary glands from *Tip30*$^{-/-}$ mice relative to age-matched *Tip30*$^{+/+}$ mice (Fig. 6*A*). In contrast, the level of cyclin B1 mRNA (Fig. 6iA), whose promoter was not bound by ERα, CIA, or TIP30 (Fig. 2*C*), was relatively normal in these *Tip30*$^{-/-}$ mammary glands. We next used an immunohistochemical analysis to evaluate c-*myc* protein expression in the *Tip30*$^{-/-}$ mammary glands. Punctate staining for c-Myc protein was detected in both-*Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mammary epithelial cells. However, 41% of the *Tip30*$^{-/-}$ mammary epithelial cells had nuclear staining for c-*myc*, whereas 27% of the *Tip30*$^{+/+}$ mammary epithelial cells had nuclear staining for c-*myc* (Fig. 6, *B* and *C*). We also examined ERα expression in these mammary glands using immunohistochemistry and Western blot analyses. The level of ERα and percentages of ERα nuclear staining in both *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mammary epithelial cells were similar (data not shown), indicating that increased c-*myc* expression is not due to an increased expression of ERα in the mammary epithelium. These results demonstrate that a lack of TIP30 results in increased c-*myc* expression in mouse mammary glands. Taking these results together, we concluded that TIP30 is a repressor of ERα-mediated transcription of the c-*myc* gene.

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DISCUSSION

ERα-mediated transcription requires coactivators that interact with ERα and enhance its transcriptional activity. Although most ER coactivators identified thus far associate with the AF-2 domain and enhance the transcriptional activity of many nuclear receptors, as well as nonreceptor activators (4, 14, 37), the unique coactivator CIA specifically enhances ERα activity independent of AF-2 function (22). In the present study, we have demonstrated the interaction between a putative tumor suppressor TIP30 and CIA and subsequently cloned the full-length cDNA encoding CIA. Our biochemical and genetic data argue strongly that TIP30 is a repressor of ERα-mediated transcription of the c-*myc* gene. Specifically, TIP30 is coimmunoprecipitated with CIA from HeLa nuclear extracts and bound to CIA *in vitro* (Fig. 1). TIP30 and CIA are associated with the c-*myc* promoter in the absence of E2 and associated with the promoter and transcribed regions in the second

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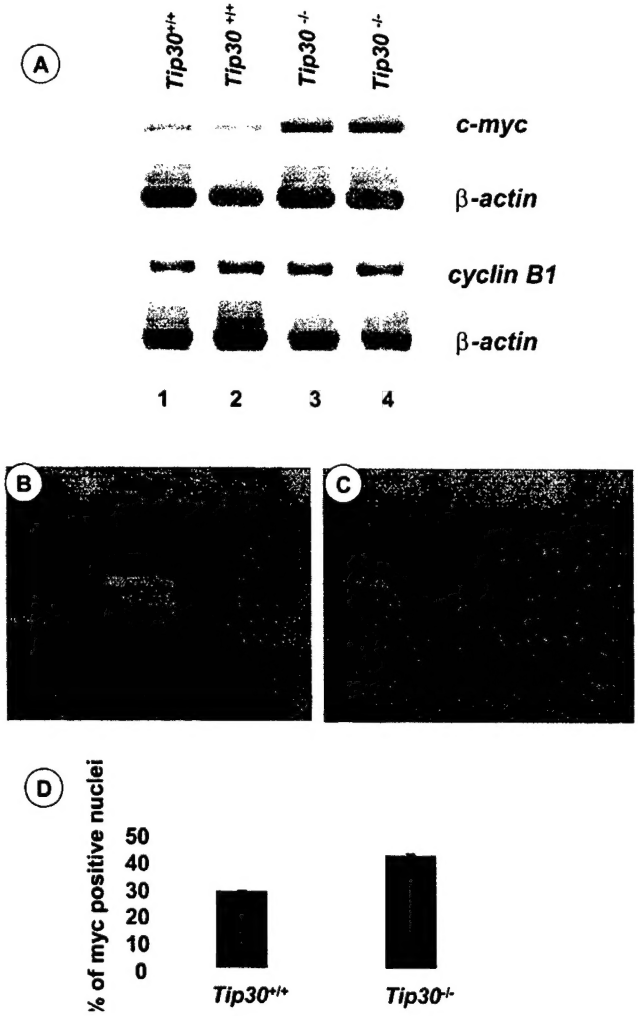

FIG. 6. **Loss of TIP30 increases c-*myc* expression in mammary glands.** *A*, loss of TIP30 increases the levels of c-*myc* mRNA in mammary glands. RNA was isolated from mammary glands of 8-week-old virgin $Tip30^{+/+}$ and $Tip30^{-/-}$ mice. RT-PCR analysis was used to monitor expression of the c-*myc*, cyclin B1, and β-actin genes. *B–D*, loss of TIP30 increases c-*myc*-stained epithelial cells in mammary glands. Representative immunostaining for c-*myc* on mammary gland sections from 8-week-old $Tip30^{+/+}$ (*B*) and $Tip30^{-/-}$ virgin mice (*C*) are shown. The *bar graph* indicates the percentage of c-*myc* nuclear-positive epithelial cells (*D*). The results represent two 8-week-old virgin mice for each genotype. About 500 epithelial cells were counted in the mammary gland section for each animal.

transcription cycle in the presence of E2, suggesting that they are present in the same transcription complexes (Fig. 2). This is further supported by the results (Fig. 3*A*) that TIP30 and CIA cooperatively inhibit ERα-mediated transcription in transient transfection assays. The fact that TIP30 and CIA were copurified in the presence of protein cross-linkers also indicates that they do not form a stable complex but may transiently interact with each other during the second transcription cycle. Finally, involvement of TIP30 in ERα-mediated transcription is also supported by the observations that a lack of TIP30 results in enhanced ERα-mediated transcription in transient transfection assays (Fig. 3) and increased c-*myc* expression in mammary glands (Fig. 4). Therefore, we conclude that interaction between TIP30 and CIA is biologically relevant, and TIP30 is a repressor of ERα-mediated transcription of the c-*myc* gene.

Although CIA is not related to any known proteins, it contains a receptor-binding motif known as the LXXLL motif and a motif consisting of Arg-Asp repeats (22). Interestingly, the Arg-Asp repeats were previously found in a putative RNA-binding component (RD) of NELF (32) that interacts with RNA polymerase II and represses transcription. Because CIA, unlike other ER-interacting coactivators, is not released during elongation (Fig. 3), it may bind to RNA via the Arg-Asp repeats to regulate elongation. It is interesting in this regard that estrogen promotes occupancy of CIA on the promoter and the coding regions of the c-*myc* gene. The precedent for this type of assembly of transcription factors is observed in galactose activation of *GAL1* and heat shock activation of *HSP82* in yeast and *hsp70* in HeLa cells. Elongation factors such as Spt5 (38) and Sug1/Rpt6 (39, 40) are associated with both promoters and open reading frames of transcribed genes in yeast. Transcription activation induces occupancy of those factors on the *GAL1* promoter and the open reading frame. Therefore, we propose that CIA may be an elongation factor that is specifically involved in the transcription of ERα-targeted genes in mammalian cells. TIP30 and CIA may represent a novel class of coregulators for ERα.

The c-*myc* gene is controlled by numerous transcription factors through upstream sequences of its P1 and P2 start sites (7, 35). Previous studies have established that ERα increases c-*myc* transcription through a noncanonical ERE element (8). The experiments in the current study were designed to first determine the role of TIP30 and CIA in ERα-mediated c-*myc* transcription. Our data clearly demonstrate that TIP30 and CIA are involved in the regulation of c-*myc* transcription. It should be emphasized that the inhibition of ERα-mediated transcription by TIP30 is not caused by a nonspecific repression, because TIP30 overexpression or loss of expression failed to alter transactivation by other nuclear receptors under the same conditions. Overall, previous (22) and current studies have suggested complicated roles of TIP30 and CIA in ERα-mediated transcription, although it remains unclear precisely how TIP30 and CIA function in this process. However, based on our data, we hypothesize that TIP30 and CIA may act as repressors of c-*myc* transcription. In this scenario, the binding of TIP30 to the c-*myc* promoter in the absence of E2 might lead to phosphorylation of the CTD of RNA polymerase II (27) during the formation of preinitiation complexes. This in turn might destabilize the association of RNA polymerase II with coactivators such as AIB1, p300, pCAF, CBP, and TRAPs (15) at the initiation step. In this regard, an inhibitor of RNA polymerase II elongation, α-amanitin, (15, 36), which inhibited the association of RNA polymerase II with the coding region of the c-*myc* gene, also abolished the association of TIP30 and CIA (Fig. 2*F*), suggesting that association of TIP30 and CIA with the coding region is RNA polymerase II-dependent. In addition, TIP30 was previously shown to phosphorylate the CTD *in vitro* (27), and the kinase-defective TIP30 mutant failed to repress ERα-mediated transcription (Fig. 4*B*). In the first transcription cycle, CIA may facilitate displacement of TIP30 from the promoter, possibly through a transient interaction with TIP30, and subsequently associate with elongation complexes. Alternatively, ERα and ERα-recruited factors could also facilitate TIP30 displacement from the promoter, with the observed TIP30-CIA interactions being more relevant to their mutual presence in transcription elongation complexes in the absence of estrogen as well as in the second cycle transcription complexes following estrogen treatment. In the second transcription cycle, CIA may cooperate with TIP30 to repress transcription. In support of this possibility, CIA was previously shown to inhibit transcription of a Gal-driven *TK* reporter when it was fused to a GAL4 DNA-binding domain, indicating that CIA may possess an intrinsic inhibitory function (22). Clearly, further studies will be required for testing this hypothesis.

Notwithstanding, we could not pinpoint the binding site for TIP30 on the c-*myc* promoter in the absence of estrogen because of the limitations in ChIP assays. Given the bewildering regulatory elements in the c-*myc* promoter and observations of RNA polymerase II holdback at the P2 promoter (35), future studies will focus on the effects of TIP30 and CIA on RNA polymerase II and the mechanism by which CIA and TIP30 regulate transcription.

The finding that TIP30 acts as a negative regulator in both unliganded and liganded ERα-mediated c-*myc* expression led us to predict that TIP30 may regulate tumorigenesis and tissue development of ERα-targeted organs (41, 42). As described in a recent report (28), we observed that a group of C57B6/129Svj hybrid mice deficient in TIP30 spontaneously developed tumors in ERα target organs including the ovary, uterus, and liver but not in the mammary gland. Recently, we have extended these studies by examining mammogenesis and tumorigenesis of cohorts of C57B6 mice deficient in TIP30 and control wild type mice. We have observed that virgin female mice lacking TIP30 exhibited ductal hyperplasia and spontaneously developed tumors in the mammary gland compared with control wild type mice.[2] Because mild hyperplasia was described previously in the mammary gland of transgenic mice overexpressing c-*myc* (9, 37, 43), the ductal hyperplasia in *Tip30*$^{-/-}$ mice could be partly due to elevated expression of the c-*myc* gene that was observed in their mammary glands (Fig. 6). This genetic evidence agrees well with a negative role of TIP30 in ERα-mediated c-*myc* transcription. However, we do not exclude the possibility that TIP30 may also regulate other ERα target genes that may also contribute to the ductal hyperplasia. Future studies should identify additional TIP30 target genes in the mammary epithelial cells and illuminate the molecular basis for TIP30 loss-facilitated tumorigenesis.

In summary, we have identified a TIP30-interacting protein as the ER-interacting coactivator CIA and revealed that TIP30 and CIA directly regulate the transcription of the c-*myc* gene. Like other ERα cofactors (14, 15), TIP30 and CIA are recruited by ERα on the c-*myc* promoter in a cyclic fashion. Concordant with its effect on ERα-mediated c-*myc* transcription, loss of TIP30 increases c-*myc* expression in the mammary gland of mice. Our studies suggest that TIP30 and CIA represent a novel class of coregulators for regulating gene expression during mammogenesis and tumorigenesis.

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